

A cytochrome *c* oxidase proton pumping mechanism that excludes the O₂ reduction site

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Abstract A redox-coupled conformational change in Asp51 of subunit I and a hydrogen-bond network connecting Asp51 with the matrix surface have been deduced from X-ray structures of bovine heart cytochrome *c* oxidase. This has provided evidence that Asp51 may play a role in the proton pumping process. However, the lack of complete conservation of a residue analogous to Asp51, the inclusion of a peptide bond in the hydrogen-bonding network and the lack of apparent involvement of the O₂ reduction site have been used as arguments against the involvement of Asp51 in the mechanism of proton pumping. This mini-review re-examines these arguments.

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1. Introduction

Cytochrome *c* oxidase is the terminal oxidase of cell respiration, reducing molecular oxygen (O₂) to water via a mechanism coupled with a proton pumping process. The proton motive force produced by the proton pumping process and O₂ reduction is energetically harnessed for production of ATP by ATP synthase [1]. The mammalian cytochrome *c* oxidase enzyme is a large multicomponent membrane protein comprising 13 different subunits, four redox-active metal sites (hemes *a* and *a*₃, Cu_A and Cu_B), three redox-inactive metal sites (Mg²⁺, Zn²⁺ and Na⁺) and several different classes of phospholipids [2]. Except for the redox-active metal sites, the roles of these components are essentially unknown. The O₂ reduction site comprises heme *a*₃ (in high-spin state in the oxidized state) and Cu_B [1]. Electrons from cytochrome *c* are accepted by Cu_A and transferred to the O₂ reduction site via heme *a* (in six-coordinated low-spin state in both oxidation states [3]). Although cytochrome *c* oxidases of bacteria are less complex, they do include the three largest subunits that are common to those of the mammalian enzymes. X-ray structures of bovine and bacterial enzymes indicate strong homology among the three-dimensional structures of these redox-active metal sites,

providing convincing evidence for the existence of a conserved reaction mechanism [4].

Spectroscopic methods have great utility for probing the structural and functional characteristics of transition metal complexes. Thus, the O₂ reduction mechanism has been extensively investigated by spectroscopic methods since the enzyme was first isolated from bovine heart [5,6]. Cu_B, located close to the O₂ binding site (heme *a*₃) is the most probable source of the second electron provided to the bound O₂, which enables a two-electron reduction of the triplet O₂ bound at heme *a*₃. The two-electron reduction is critical for O₂ reduction, since a single electron reduction of O₂ is energetically unfavorable [7]. Thus, the proximity of the two transition metals in the O₂ reduction site, first deduced by electron paramagnetic resonance (EPR) investigations [8], strongly suggests that the O₂ bound at heme *a*₃ is readily reduced to a form of a bridged peroxo intermediate (Fe³⁺_{a3}–O–O–Cu²⁺_B) [7]. Therefore, it was expected that the oxygenated form, Fe²⁺_{a3}–O₂, is unlikely to be detected during the course of O₂ reduction by the enzyme. However, time-resolved resonance Raman spectroscopic investigations showed that the initial intermediate in the O₂ reduction by the enzyme is an oxygenated form in which O₂ is bound at Fe²⁺_{a3} in an end-on fashion, similar to the mode by which hemoglobins and myoglobins bind O₂ [9]. This finding indicates that essentially no interaction occurs between Cu_B and the O₂ bound at Fe_{a3}, an observation which is apparently in conflict with the clear magnetic interaction observed between Cu_B and Fe_{a3} in the oxidized state [8]. This apparent conflict has been resolved by subsequent X-ray structural analysis of the O₂ reduction site in the reduced state at 2.35 Å resolution, which indicates that Cu_B is coordinated by three histidine imidazoles in symmetric trigonal planar geometry with the Cu_B in the geometric center [10]. It is well known that trigonal planar cuprous complexes are quite stable. Thus, Cu_B in the reduced state is a very poor ligand acceptor and a very poor electron donor, indicating that in the reduced state of the enzyme the role of Cu_B⁺ is to provide a stable oxygenated complex similar to those of the oxygen transporting proteins that do not reduce O₂.

Three-dimensional conformational changes are necessary in order for cytochrome *c* oxidase to pump protons. A minimal structural requirement for a redox-driven proton pump such as cytochrome *c* oxidase is described as follows: in the resting oxidation state, a proton-accepting group with a pK_a value significantly higher than the pH of the matrix space is capable of proton uptake only from the matrix space in this oxidation

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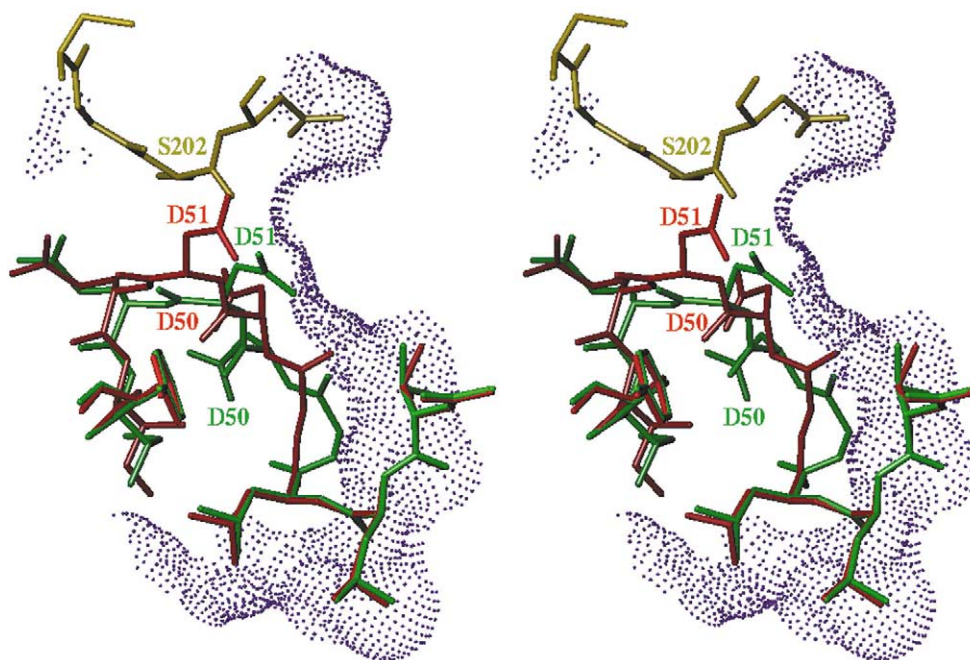


Fig. 1. Redox-coupled conformational change in the segment from Gly49 to Asp55 of subunit I of bovine heart cytochrome *c* oxidase at 2.3/2.35 Å resolution in the fully oxidized/reduced states, respectively. The conformation of the segment in the fully oxidized state is stereoscopically shown in red and that in the fully reduced state in green. The yellow structure denotes subunit II. The accessible surface for the fully oxidized state is indicated by dots.

state. Upon reduction of the enzyme, the pK_a of the proton-accepting group is lowered significantly while it changes conformation, becomes accessible to the intermembrane side, and releases protons. Significant conformational changes are necessary in order for changes in pK_a and the intermembrane accessibility of the proton-accepting group to occur. Thus, in order to deduce the mechanism of the proton pumping process of cytochrome *c* oxidase, a redox-coupled conformational change should be detected in the X-ray structures of this enzyme. It was suspected that the conformational change could be extremely small and difficult to detect. Thus, we have sought to improve the resolution of X-ray structures of bovine heart cytochrome *c* oxidase in various oxidation and ligand binding states. In 1998, we reported the observation of a redox-coupled conformational change in an aspartate residue (Asp51) and a hydrogen-bond network connecting the residue with the matrix space, which provided a good indication that Asp51 could be the proton-accepting group responsible for driving the proton pumping process [10]. Previously proposed mechanisms [1] for the proton pumping process have consistently discounted the potential role of Asp51 for reasons to be discussed in this minireview.

2. The redox-coupled conformational change of Asp51 in X-ray structures of bovine heart cytochrome *c* oxidase at 2.3/2.35 Å resolution in the fully oxidized/reduced states

As shown in Fig. 1, Asp51 of subunit I of bovine heart cytochrome *c* oxidase in the fully oxidized state is completely buried inside the protein such that it is not accessible to water in the intermembrane phase. The molecular surface to which the water molecules in the intermembrane phase are accessible is shown by the dotted surface. On the other hand, X-ray structures indicate that, upon reduction of the enzyme,

Asp51 moves toward the molecular surface and is exposed to the intermembrane phase [10]. In the oxidized state, the carboxyl group of Asp51 is hydrogen-bonded to the peptide amide groups (N–H) of Ser205 and Tyr440 and two hydroxyl groups of Ser205 and Ser441. The carboxyl group does not hydrogen-bond to a water molecule in the oxidized state. Thus, the polarity of the environment of the carboxyl groups is expected to be similar to that of ethanol. It is well known that pK_a of a carboxyl group is greatly influenced by solvent polarity [11]. The pK_a value of acetic acid is 10.0 in ethanol. Thus, the carboxyl group of Asp51 in the oxidized state is likely to be protonated. In the oxidized state, Asp51 is connected with the molecular surface facing the matrix space, via a hydrogen-bond network connected to a water channel, as shown in Fig. 2, which acts as a source of protons from the matrix space. Thus, in the oxidized state, Asp51, with its high pK_a proton-accepting group, can take up protons from the matrix space. All hydrogen bonds of the network, with the exception of the bond to the Ser441 hydroxyl group, are broken upon reduction of the enzyme. In the reduced state of the enzyme, the carboxyl group is found in an aqueous environment wherein it is expected to have a pK_a of about 4.5 and thus to be capable of releasing protons to the intermembrane space [10]. These structural findings provide convincing evidence that Asp51 is responsible for driving the proton pumping process.

3. Discussion of the function of the H pathway

3.1. Asp51 is not conserved completely

As described above, Asp51 of bovine heart cytochrome *c* oxidase is conserved only among members of the animal kingdom. Bacterial and plant terminal oxidases do not have aspartate or glutamate at the corresponding site. On the other

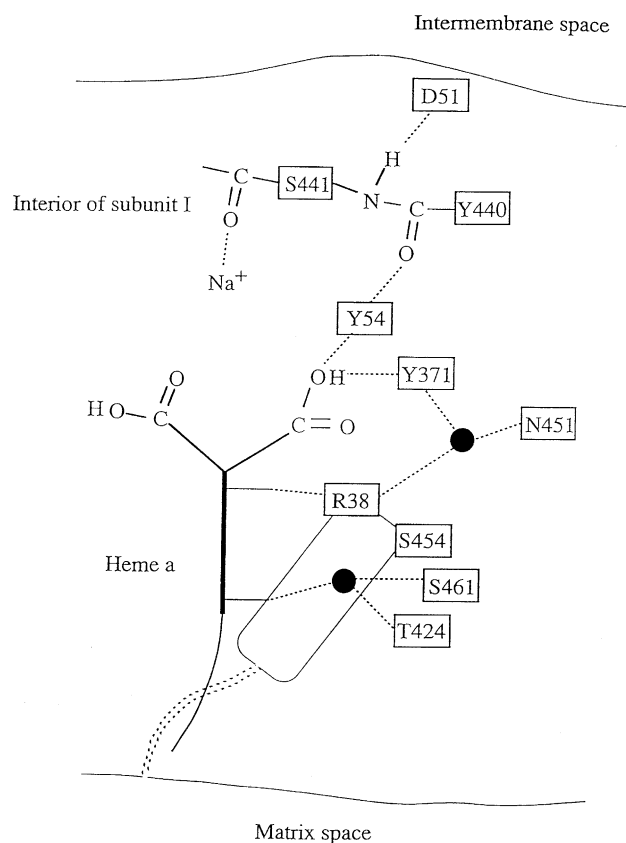


Fig. 2. A schematic representation of H pathway in the oxidized state. Dotted lines denote hydrogen bonds. The dark circles indicate fixed waters. A thick stick denotes a side view of the porphyrin plane of heme *a* and thin sticks (from top to bottom) are the side chains, propionates, formyl and hydroxyfarnesylethyl.

hand, the amino acid residues included in the O_2 reduction site are completely conserved. Proton pumping is as important physiologically as the O_2 reduction in this enzyme. The fact that the amino acid residues of the O_2 reduction site are completely conserved tends to make investigators believe that it must be involved in the mechanism of proton pumping. However, the essential differences between the two chemical processes should be examined in greater detail. O_2 reduction is a very complex chemical process that includes redox chemistry for the activation of triplet oxygen and O–O bond cleavage without the release of active oxygen species from the O_2 reduction site. On the other hand, a proton pumping mechanism may be envisioned as a chemically simple process comprised of multiple protonation/deprotonation steps. For the O_2 reduction process, the high-spin heme- Cu_B system could simply be the most effective biological system available and early evolutionary optimization of this system has necessitated complete conservation of the amino acid residues in the O_2 reduction site. On the other hand, many amino acid functional groups, prosthetic groups and even water molecules are able to transfer protons. Thus, a lack of absolute homology between these protonatable groups would not be expected to abolish proton pumping activity. This is the case for the hydrogen bond networks for proton transfer for water formation (K and D pathways) [12]. No amino acid residues in the pathway are completely conserved. It has been claimed that completely different sets of amino acid residues could function

in a physiologically identical process [12]. At present, X-ray structures of three bacterial oxidases have been solved at resolutions better than 2.8 Å [13–15]. Two bacterial *aa3*-type cytochrome *c* oxidases have a glycine at the point corresponding to the site of Asp51 in bovine cytochrome *c* oxidase [13,15]. A small cavity capable of trapping a water molecule is located between the glycine and the peptide bond in the hydrogen-bond network. The glycine–water combination is expected to be capable of transferring protons from the hydrogen-bond network to the periplasmic phase with a conformational change controlling the accessibility of the water molecule to the aqueous phase. On the other hand, the *ba3*-type cytochrome oxidase of an extreme thermophilic bacterium does not have a pathway homologous to the H pathway, but it does contain another hydrogen-bond network which extends across the enzyme molecule [14]. These X-ray structural results also suggest variety in the structure of proton transfer pathways. Therefore, the fact that Asp51 is not completely conserved does not provide decisive evidence against the proposed proton pumping function of Asp51.

3.2. Proton transfer through a peptide bond

The fact that a peptide bond is included in the hydrogen-bond network also contributes to the lack of acceptance of the involvement of Asp51 in the proton pumping process, because peptide bonds have not typically been observed in the hydrogen-bonding networks of other enzyme systems. However, proton transfer through a peptide bond is possible if imidic acid intermediate ($-C(OH)=N^+H-$) formation [16] and keto–enol tautomerization are taken into account. Asp51 hydrogen-bonded to the peptide amide group will take up protons readily from the imidic acid since its pK_a is quite high in the interior of the protein, as described above. The resulting enol form of the peptide ($-C(OH)=N-$) is rapidly transformed to the keto form ($-CO-NH-$), since the latter is much more stable than the former and the conformational change accompanied by the transition is almost negligible. The peptide bond thus imparts unidirectionality to the proton transfer.

One might claim that if peptide bonds could serve in proton transfer pathways, the transmembrane helices of most membrane proteins would be effective proton transfer pathways. In fact, such a proposal was reported long ago [17]. However, proton transfers through transmembrane helices at physiologically significant velocities are unlikely to occur since the formation of an imidic acid intermediate is an energetically unfavorable process. Thus the reverse reaction is equally possible via peptide bonds in transmembrane helices, in which case proton transfer rates would decrease exponentially with the number of peptide bonds in a helix. Furthermore, hydrophobic side chains in the transmembrane region of the membrane protein are expected to have the effect of lowering the rate of the forward process and accelerating the reverse process. On the other hand, the peptide bond of the H pathway is coupled to a clearly irreversible step by Asp51, which removes protons from the imidic acid in the oxidized state and releases protons into the intermembrane space in the reduced state. Furthermore, the donation of protons to the peptide carbonyl group is also an essentially irreversible process, as described below. The preceding discussion indicates that within the proton pumping mechanism, proton transfer through a peptide bond is a chemically feasible event.

3.3. The driving element of proton pumping

The overall process of the cytochrome *c* oxidase reaction, which includes the proton pumping process, is driven by reduction of O_2 to water. Thus, it seems reasonable to assume that proton pumping (an energy-requiring process) is coupled to O_2 reduction at the O_2 reduction site. However, time-resolved resonance Raman investigations have shown that the unexpectedly stable oxygenated state ($Fe_{a3}^{2+}-O_2$) and the unexpectedly unstable peroxide intermediate give rise to $Fe_{a3}^{5+}=O^{2-}$ and $Fe_{a3}^{4+}=O^{2-}$ species [9]. The results indicate that a four-electron reduction of O_2 occurs from the oxygenated state ($Fe_{a3}^{2+}-O_2$) to the oxide level (O^{2-} and H_2O), which requires high oxidation states of heme Fe_{a3} (Fe^{5+} or Fe^{4+}). This four-electron reduction occurs at a very fast rate without the release of active oxygen species. This result indicates that the high oxidation states of Fe_{a3} are produced by the large free energy change accompanied by the O_2 reduction. The high oxidation state of Fe_{a3} is capable of essentially irreversible uptake of electrons from heme *a* and Cu_A (since Cu_A is in rapid redox equilibrium with heme *a*). The irreversible electron transfer processes can thus drive the energetically unfavorable process of proton transfer from the matrix space to Asp51 via the peptide bond. In the absence of metal ligands, the redox potentials of the four redox-active metal sites are essentially identical [18]. However, CO and O_2 increase the redox potential of Fe_{a3} and Cu_B and thus greatly stabilize the reduced states. Therefore, in the presence of O_2 , electron transfer from Cu_A /heme *a* to the Fe_{a3}^{3+} and Cu_B^{2+} sites is essentially irreversible. Thus, under turnover conditions or under aerobic conditions, electron transfer from Cu_A /heme *a* sites to the O_2 reduction site is always likely to be accompanied by a free energy change large enough to drive active proton transfer to Asp51.

The proton ejection function of bovine heart cytochrome *c* oxidase during the complete oxidation of the fully reduced enzyme reconstituted in proteoliposomes with O_2 has been reported from two different research groups [19,20]. The experimental results clearly indicate that one proton equivalent per enzyme molecule is ejected during the process. (However, following the reinterpretation of the results of both investigations, it was determined that a maximum of two proton equivalents is ejected during the process [19,20]). In any case, if it is assumed that four proton equivalents are pumped by the reduction of one equivalent of O_2 , the reported results are clearly in conflict with the proposal that direct coupling occurs between the O_2 reduction process and the proton pumping process, since complete oxidation of the fully reduced enzyme with O_2 includes the entire process of O_2 reduction to water. Investigations of proton ejection during the reductive phase reported by Verkhovsky et al. [20] strongly suggest that the proton pumping is coupled to the internal electron transfer process from the Cu_A /heme *a* sites to the O_2 reduction site (Fe_{a3}^{3+} and Cu_B^{2+} states).

Certain bacteria have terminal oxidases, known as quinol oxidases, which lack Cu_A and reduce O_2 to water in a process coupled to proton pumping wherein electrons are accepted directly from quinol molecules. Quinol oxidases have three redox-active sites: a low-spin heme, a high-spin heme and Cu_B , which are essentially conserved in the heme-copper terminal oxidase superfamily that includes cytochrome *c* oxidases and quinol oxidases. Interestingly, certain quinol oxidases have low-spin heme B and high-spin heme O. Heme O

is a derivative of heme A which has a methyl group instead of the formyl group [1]. Each of these terminal oxidases has a low-spin heme in addition to the high-spin heme which provides the site for O_2 binding. The fact that the low-spin heme is conserved within the superfamily strongly suggests a physiological role of the heme other than acting as a simple electron transfer mediator. Such a role could be a contribution to the proton pumping mechanism.

3.4. Other arguments against Asp51 playing a role in the proton pumping process

X-ray structures at 2.30–2.35 Å resolution are unable to indicate the electron density of a hydrogen atom. However, the direction of hydrogen bond structure can often provide a clear indication of protonation state changes of acidic amino acid residues. FTIR spectroscopy is a powerful tool for examination of the protonation/deprotonation state change, since the antisymmetric vibration of a protonated carboxyl group gives rise to a band near 1740 cm^{-1} whereas the analogous band of a deprotonated carboxyl group is found near 1580 cm^{-1} [21,22]. The FTIR difference spectra of bovine heart cytochrome *c* oxidase in the oxidized state vs. the reduced state exhibit a positive peak at 1738 cm^{-1} and a negative peak at 1580 cm^{-1} [22]. The former band is upshifted upon D_2O exchange [23]. These positive and negative peaks are not detectable in bacterial *aa3*-type cytochrome *c* oxidases [22]. Furthermore, no other acidic amino acid in bovine heart cytochrome *c* oxidase shows a redox-coupled conformational change suggestive of a change in the protonation state of the carboxyl group [10].

It has been proposed that there is a contribution of electrostatic interactions between Cu_A and the Asp51 carboxyl group because Cu_A is located fairly close to Asp51 (6–7 Å) [24]. However, in the oxidized state, the carboxyl group is essentially in the fully protonated state, while in the reduced state, the Cu_A site does not carry a net charge. The positive charge in the oxidized state may contribute to the stabilization of the conformation of the carboxyl group prior to protonation.

All members of the heme-copper terminal oxidase superfamily have two hydrogen bond networks connecting the matrix surface with the O_2 reduction site. Replacement via site-directed mutagenesis of the hydrogen-bond forming amino acids in the D pathway with non-polar amino acids completely abolishes electron transfer activity together with its proton pumping function [25]. These mutant enzymes receive electrons readily from cytochrome *c* to form the fully reduced enzyme. However, upon reduction, when these mutant enzymes react with O_2 , they do not transfer four electron equivalents to form the fully oxidized enzyme on a physiologically relevant timescale [25]. The observation of absorption spectral changes during this process suggests that transfer of the last two electron equivalents to the O_2 reduction site is impaired by the mutations in D pathway [25]. Since these mutations abolish the proton pumping process, it may be reasonable to propose that the D pathway transfers protons to be used for proton pumping at or near the O_2 reduction site, as well as for water formation. This proton pumping at or near the O_2 reduction site which includes completely conserved histidine ligands to the two metal ions is consistent with the widely accepted view that structures for the proton pumping are completely conserved [26]. However, if protons are pumped at or near the O_2 reduction site, the O_2 reduction site must

have a functional group capable of sorting the protons to be pumped from the protons used for water formation, as has been suggested earlier [27]. The O₂ reduction site does not appear to have such a functional group. The structure of the O₂ reduction site and the possible involvement of heme *a* in the proton pumping as described above strongly suggest the alternative interpretation that strict coupling occurs between the proton transfer process for water formation (through D pathway) and the electron transfer process from heme *a* to the O₂ reduction site which is coupled to the proton pumping process.

The function of the H pathway has been examined by site-directed mutagenesis of residues within the H pathway of the two bacterial cytochrome *c* oxidases that lack an Asp or Glu residue analogous to Asp51 (bovine) [28,29]. Mutation of amino acids in the wall of the water channel in the H pathway with amino acids similar to or smaller than those of the wild-type enzyme did not affect the proton pumping function, presumably because these mutations would not be expected to interfere with the water migration in the water channel. In bovine cytochrome *c* oxidase, mutation of Arg38 (which is hydrogen-bonded to the formyl group of heme *a*) to Met decreases the O₂ reduction activity without affecting the ratio of pumped protons to electron equivalents transferred to the O₂ reduction site [30]. The loss of the hydrogen bond between the formyl group and Arg38 present in the wild-type enzyme is expected to lower the electron transfer rate due to a significant change in redox potential. However, methionine is unlikely to abolish the proton transfer process since the thioether group of methionine is able to participate in proton transfer [31]. Thus, these results do not provide decisive evidence against the proton pumping process occurring with source protons obtained via the H pathway.

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